Metagenomic Analysis of Uterine Microbiota in Postpartum Normal and Endometritic Water Buffaloes (*Bubalus bubalis*)

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Abstract: In Indian subcontinent the water buffalo (*Bubalus bubalis)* is one of the important livestock animals. As in cows, postpartum infection like endometritis in dairy buffaloes is major cause for the economic loss in the dairy industries. Till date, there is no study regarding metagenomic analysis of bacterial population of postpartum endometritic buffaloes. The purpose of this study was to identify and compare the uterine bacterial composition in normal and endometritic postpartum buffaloes using 16S rDNA cloning, which was a type of culture-independent methods. A total of 151 cloned plasmids for 16S rDNA from both normal and endometritic uterine samples were sequenced. Cloning library of 16S rDNA revealed clear cut difference between bacterial populations of normal and endometritic postpartum buffaloes. Cloned sequences were assigned to five major groups and one uncultured group. The five major groups include- *Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria*, and *Tenericutes*. Major cloned sequences from normal status endometrium were affiliated to phylum *Proteobacteria,* and most of the sequences showed high degree of similarity with bacteria *Haemophilus felis*. Most of the sequences from cloned library of endometritic status samples were affiliated to phylum *Proteobacteria* and *Tenericutes.* The most prevalent bacteria found in endometritic samples were *Psychrobacter* sp. PRwf-1, *Psychrobacter pulmonis*, *Ureaplasma diversum* strain T95 and *Ureaplasma diversum* strain A417. A major number of cloned sequences from both normal and endometritic samples were assigned to uncultured group. The present data showed bacterial population of postpartum normal and endometritic buffaloes and also described the presence of various types microbiota in uterine samples.

Keywords: Buffalo, endometritis, 16S rDNA cloning, bacterial population.

INTRODUCTION

The period between parturition to complete uterine involution is called as postpartum period [1]. In the postpartum period, the chances for uterine infections are more because of the opening of cervix. The incidence rate of uterine infection in buffaloes has been found to be much higher than in cows [2, 3]. Therefore our rationale of this study to identify the types of bacterial population present in the uteri of the postpartum buffaloes. Inflammation of endometrium is called as endometritis. In buffaloes endometritis is the most frequent cause for the infertility. Incidence of endometritis is high in buffaloes 9.07-67.11% [4]. The main reason for endometritis is nonspecific opportunist pathogens that contaminate the uterus during the periparturient period. During the first week of postpartum, the rate of isolation of bacteria from uterine tract of the buffaloes was high, followed by two to four weeks of calving. *E. coli* was the most predominant isolates followed by *S. aureus* then *S. pyogenes* [5]. There are no full-fledged studies about the presence of micriobiota in the uteri of postpartum buffaloes. Even though *E. coli* and *A. pyogenes* have significant role in the postpartum infections of cows because of the presence in the contaminated uterus [6-9], the other bacteria like *Fusobacterium necrophorum*, *Prevotella melaninogenicus*, *Bacteroidetes* spp., *Pseudomonas* spp., *Streptococcus* spp., and *Staphylococcus* spp., etc., have also been isolated from infected uteri of cows which may also responsible for the postpartum infections in cows [7, 10]. Previously the identification of bacteria and their characterization in uteri of bovine was mostly relied on cultivation of uterine swab or secretions. Because of the limitations associated the culture dependent methods, they have been underestimated the complexity microbial population [11, 12]. Therefore the culture-independent methods, as proposed by metagenomics [13, 14], are now fundamental in studying and understanding the physiology, genetics, and community ecology structure of the unseen majority. The cloning and sequencing of 16S rRNA gene fragments is one of the culture independent methods to be used for the metagenomic analysis. The metagenomic analysis of uterine microbiota by 16S rRNA cloning in postpartum healthy and metritic cows has shown the presence of large number of bacterial populations [15].

Some of the uterine microbial population of postpartum buffaloes have been identified by culture

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dependent methods, but there is no culture independent studies regarding to identify the majority of bacteria in the buffaloes uterine after parturition. In this study we have tried to identify the microbiota of postpartum normal and endometritic buffaloes uterine fluids by using culture independent methods like 16S rRNA gene fragment cloning.

MATERIALS AND METHODS

Collection of Uterine Fluid from the Postpartum Buffaloes

Uterine fluid was collected from water buffaloes (*Bubalus bubalis*) from the dairy farm, NDRI, Karnal, India. The uterine fluid was collected after 21 days of parturition. Uterine fluids have been collected from 3 buffaloes which were at normal state and from 3 buffaloes which were at endometritis state. Uterine fluid was collected from the buffaloes by using blue sheet aseptically into 15ml sterile plastic tubes (Tarsons, India). Clinical endometritis was characterized as the presence of a purulent uterine discharge detectable in the vagina 21 days or more postpartum and described earlier [16].

Extraction of Bacterial DNA from Uterine Fluid

The total bacterial DNA was isolated from the uterine fluid by using bacterial DNA isolation kit (GenElute™ Bacterial Genomic DNA kit, Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Total DNA was eluted in 200 µL of elution solution provided in the kit. The concentration and purity was checked by optical density using NanoPhotometer (Version 2.2, IMPELEN, Germany) at 260 and 280 nm wavelengths. The integrity was checked in 1.2% (wt/vol) agarose gel (0.5µg/ml ethedium bromide) electrophoresis and visualized with Gel Doc (***Molecular Imager*** Gel Doc XR System, Bio-Rad, Hercules, California).

PCR Amplification of 16S rRNA Gene Fragments

The PCR (polymerase chain reaction) of bacterial 16S rRNA fragment genes from metagenomic DNA extracted from postpartum buffaloes uterine fluid was performed using the primers 27F/1522R [17] (Table **1**). The parameters for PCR were initial denaturation for 2 min at 94°C, followed by 34 cycles of denaturation (94°C for 30 s), annealing (58°C for 50 s), extension (72°C for 1 min), and a final extension at 72°C for 7 min and pause at 4^0C [15]. The bands of PCR amplified products were visualised under in Gel Doc (Molecular Imager Gel Doc XR System, Bio-Rad, Hercules, California) after agarose gel (1.2%) electrophoresis. The bands which were nearly 1500 bps considered as positive one. Purification of PCR products from agarose gel was done by using GeneJET™ Gel Extraction Kit (Fermentas Life Sciences, EU).

Cloning and Construction of 16S rRNA Gene Clone Library

Purified PCR products of 16S rRNA gene were cloned by using pGEM-T vector (pGEM®-T Vector System I, Promega, USA). The products were ligated into pGEM-T vector according to manufacturer's instructions. Then the ligated plasmid was transformed into chemically competent *E. coli* (XL blue strain) cells. 5µl of ligated product was added in a vial containing 100µl of competent cells and kept on ice for 15-20 min. Then heat shock was given at 42^0C for 90 seconds. Then immediately kept on ice for 2 min and then 250µl of SOC (Super Optimal broth with Catabolite repression) was added and incubated in shaking incubator for 1h at 37^0C with 225rpm. Total solution containing transformed competent cells were allowed to grow aerobically for overnight at 37^0C on Luria-Bertani (LB) Agar media (Himedia, India) containing ampicillin (50µg/ml) (Himedia, India) and X-gal (40µg/ml) (Fermentas Life Sciences, USA). After overnight incubation, individual white colonies were randomly picked and placed into 5ml LB broth containing ampicillin (50µg/ml), grown aerobically at 37^0C for 16h in a shaking incubator at 225rpm speed. Plasmids were isolated from the *E. coli* cells by using GeneJET™ Plasmid Miniprep Kit (Fermentas Life Sciences, EU) and insertion of 16S rRNA gene fragment into vector was confirmed by PCR. The positive plasmids were sequenced by using primer of T7 promoter. The remaining plasmids were stored at -

Table 1: Primers that were Used to Amplify the 16S rDNA of Bacterial DNA Isolated from Postpartum Buffaloes

Primer	Sequence (5'→3')	Reference
27F	AGAGTTTGATCMTGGCTCAG	Giovannoni *et al.* (1991)
1522R	AAGGAGGTGATCCANCCRCA	Giovannoni *et al.* (1991)

20^0C. One strand of DNA insert was sequenced, which is enough for the taxonomic identification of cloned 16S rRNA gene fragments obtained using BLAST (Basic Local Alignment Search Tool: http://blast.ncbi.nlm.nih.gov) search function [18].

Construction of Phylogenetic Tree

The evolutionary relationship between the buffaloes intra uterine microbiota using 16S rRNA gene clone libraries was done by MEGA4 software. The BLAST algorithm was used to compare the sequences obtained by cloning and sequencing of 16S rRNA gene fragments with sequence stored in Gen Bank using BLAST algorithm [18]. All the sequences were aligned by ClustalW version 2.0 (http://www.ebi.ac.uk/tools/msa/clustalw2) [19]. The conserved sequence from all cloned sequences was imported to the MEGA 4 software. The phylogenetic tree was constructed based on these sequence alignments using the neighbour-joining algorithm [20]. Evolutionary distances were computed using the Jukes-Cantor method [21].

Statistical Analysis

The statistical analysis for the bacteria commonly present in both normal and endometritic clone libraries were done by using Z-test. The statistical analysis was also done between the same groups of normal and endometritic clone libraries by using Z-test (http://in-silico.net/tools/statistics/ztest).

RESULTS

Characterization of Buffaloes as Normal and Endometritic

The buffaloes status was characterized as normal or endometritic based on the absence or presence of flecks in the uterine fluid collected from the postpartum uterine of the buffaloes after 21 days of parturition (Figure **1**).

Relation between Intrauterine Bacterial Communities and Phylogenetic Analysis

Total 151 clones from two libraries (55 and 96 clones from normal and endometritic status library, respectively) were screened. The partial sequence of 151 16S rRNA clones was obtained to identify the major bacteria present in the uteri of the postpartum normal and endometritic buffaloes. Based on the BLAST searches and phylogenetic analysis the clone sequences were fell into five major groups of bacteria domain, that are *Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria*, and *Tenericutes* (Figures **2**, **3**) and we also observed a group of uncultured

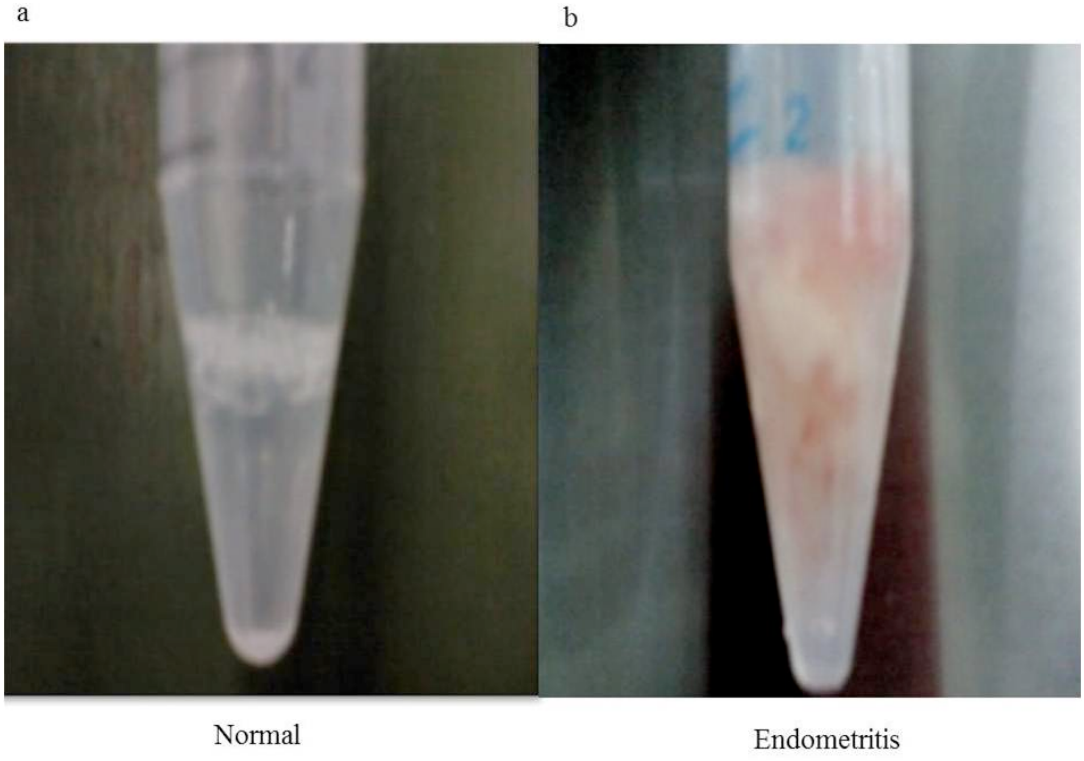

Figure 1: Characterization of buffaloes based on uterine fluid collected after 21 days of parturition. (**a**) The fluid which was not having flecks considered as normal status, (**b**) the fluid which was having flecks considered as endometritis.

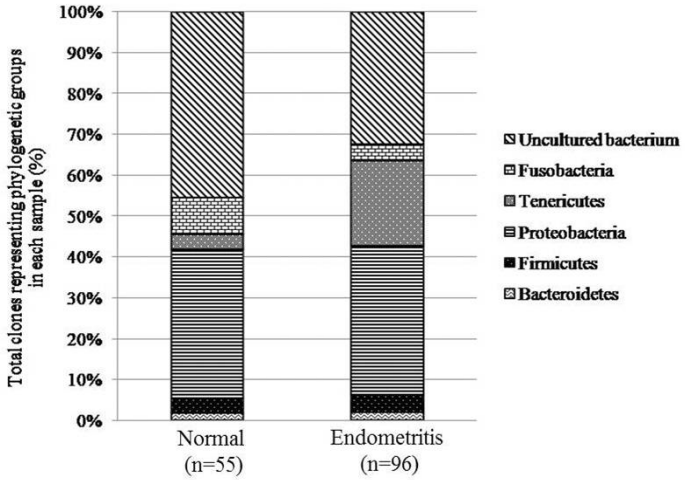

Figure 2: Stacked bars showing the bacterial group-level compositions of the uteri of normal and endometritic postpartum buffaloes.

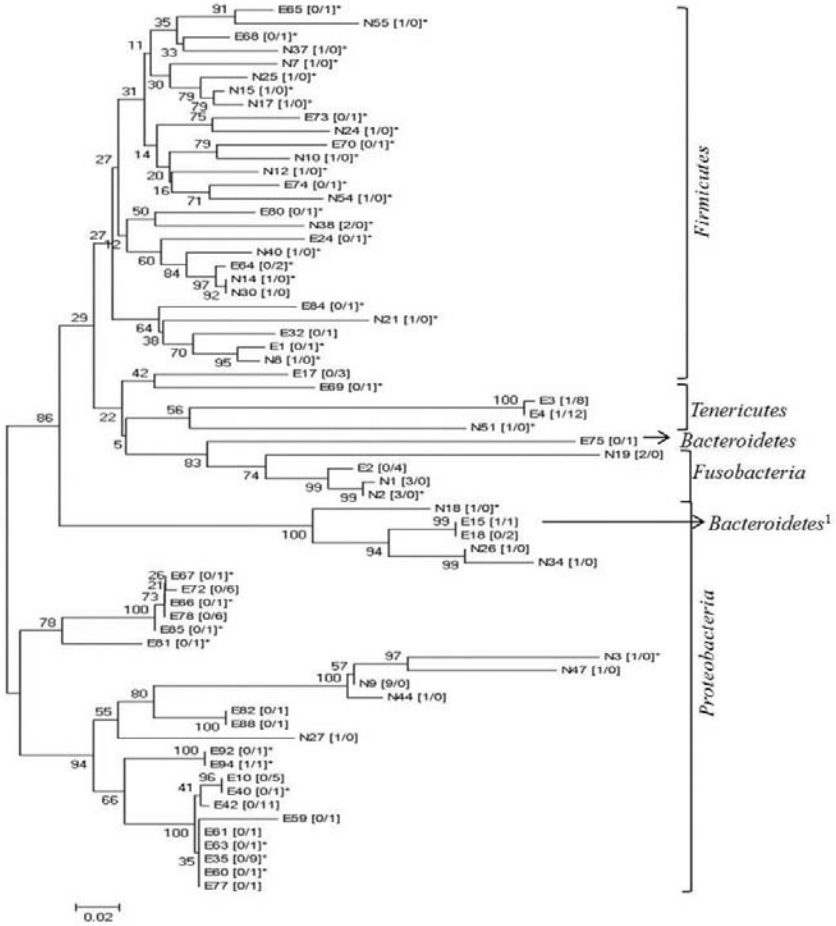

Figure 3: Phylogenetic tree of the bacterial groups identified from clone libraries from uteri of normal (n = 3) and endometritic (n = 3) postpartum buffaloes showing their affiliations. The evolutionary history was inferred using the neighbor-joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances (computed using the Jukes-Cantor method) used to infer the phylogenetic tree. Numbers at the nodes indicate bootstrap values out of 1,000 resamplings. Numbers of clones within each operational taxonomic unit (OTU) identified in the normal status and endometritic status libraries, respectively, are indicated between square brackets [normal/endometritic].

*Clones that showed high similarity with uncultured group of bacteria.

[1]Based on BLAST search the clone sequence (E15) is matched with the bacteria related to the group *bacteroidetes*, but the phylogenetic tree analysis shown that this sequence have high identity with the sequence (E18) which was matched with the bacteria related to the *Proteobacteria* group.

Table 2: Distribution of 16S rRNA Gene Sequences Obtained from Normal and Endometritic Buffalo Uterine Samples

Clone Name	Sequence affiliation (NCBI accession no.)[1]	Clones identified, n* (% of clones)[2]	
		Normal	Endometritis
	1) *Bacteroidetes*:	**1(1.82)**	**2(2.08)**
E15	*Bacteroides ureolyticus* strain R-37890 (FN401327.1)	1(1.82)	1(1.04)
E75	*Sphingobacterium* sp. HaLB8 (HM352374.1)	0(0)	1(1.04)
	2) *Firmicutes*:	**2(3.63)**	**4(4.16)**
E17	*Streptococcus uberis* 0140J (AM946015.1)	0(0)	3(3.12)
N40	*Lachnospiraceae bacterium canine* oral taxon 037 clone OD066 (JN713203.1)	1(1.82)	0(0)
N30	*Clostridium nexile* DSM 1787 (NR_029248.1)	1(1.82)	0(0)
E32	*Filifactoralocis canine* oral taxon 001 clone OB055 (JN713152.1)	0(0)	1(1.04)
	3) *Proteobacteria*:	**20(36.36)**	**35(36.46)**
E10	*Psychrobacter*sp. PRwf-1 (CP000713.1)	0(0)	5(5.21)
E59	*Psychrobacter*sp. 22 (FJ613604.1)	0(0)	1(1.04)
E61	*Psychrobacter*sp.BSw21684 (JQ069959.1)	0(0)	1(1.04)
E42	*Psychrobacter pulmonis* strain KOPRI24933 (EF101551.1)	1(1.82)	11(11.46)
E72	*Pseudomonas psychrophila* strain HA-4 (JQ968688.1)	0(0)	6(6.25)
E77	*Psychrobacterfaecalis* strain SCSGAB0010 (JX315290.1)	0(0)	1(1.04)
E78	*Pseudomonas* sp. P4 (2010) (HM196356.1)	0(0)	6(6.25)
E82	*Yersinia similis* partial 16S rRNA gene, strain Y239 (AM182407.1)	0(0)	1(1.04)
E88	*Yersinia pestis* A1122 (CP002956.1)	0(0)	1(1.04)
N3	*Pasteurellaceae bacterium Baika3* (HM626621.1)	1(1.82)	0(0)
N4	*Pasteurellamairii*strain 9801/75 (AY431032.1)	1(1.82)	0(0)
N9	*Haemophilusfelis* strain ATCC49733 (NR_025073.1)	9(16.36)	0(0)
N6	*Aggregatibactersegnis canine* oral taxon 093 clone OE003 (JN713257.1)	1(1.82)	0(0)
N44	*Pasteurellacanis canine* oral taxon 273 clone ZJ072 (JN713438.1)	1(1.82)	0(0)
N45	*Haemophilusparasuis* strain HS1079 (FJ667960.1)	1(1.82)	0(0)
N47	*Actinobacillusseminis* strain CCUG 27187 (NR_042872.1)	1(1.82)	0(0)
N48	*Bisgaard* Taxon 17 (AF024529.1)	1(1.82)	0(0)
E18	*Campylobacter hominis* ATCC BAA-381 (CP000776.1)	0(0)	2(2.08)
N26	*Campylobacter* sp. canine oral taxon 011 clone ZJ010 (JN713171.1)	1(1.82)	0(0)
N34	*Campylobacter concisus* strain UNSWCD (GQ167662.1)	1(1.82)	0(0)
N27	*Neisseria canis canine* oral taxon 137 clone OK030 (JN713302.1)	1(1.82)	0(0)
	4) *Tenericutes*:	**2(3.63)**	**20(20.83)**
E3	*Ureaplasma diversum* strain T95 (JN935894.1)	1(1.82)	8(8.33)
E4	*Ureaplasma diversum* strain A417 (NR_025878.1)	1(1.82)	12(12.5)
	5) *Fusobacteria*:	**5(9.09)**	**4(4.16)**
E2	*Fusobacterium varium* (AB640694.1)	0(0)	4(4.16)
N1	*Fusobacterium* sp. CSL-7530 (EU597748.1)	3(5.45)	0(0)
N19	*Streptobacillu s*sp. canine oral taxon 370 clone 2B078 (JN713542.1)	2(3.63)	0(0)
	6) Uncultured:	**25(45.45)**	**31(32.29)**
E1	Uncultured bacterium clone EAC_1aaa02e08 (EU774679.1)	0(0)	1(1.04)
E24	Uncultured bacterium clone IR aaa02h01 (EU474649.1)	0(0)	1(1.04)
E35	Uncultured bacterium clone A-18 (HQ860486.1)	0(0)	9(9.37)
E40	Uncultured bacterium clone 1103200832522 (EU845713.1)	0(0)	1(1.04)
E60	Uncultured bacterium clone 1103200828900 (EU845467.1)	0(0)	1(1.04)
E63	Uncultured bacterium clone HWGB-66 (JQ684324.1)	0(0)	1(1.04)
E64	Uncultured bacterium clone KO1_aai44c07 (EU461105.1)	0(0)	2(2.08)
E65	Uncultured bacterium clone BH2_aao23c02 (EU466407.1)	0(0)	1(1.04)
E67	Uncultured bacterium clone calf32_10wks_grp1_F02 (GQ448226.1)	0(0)	1(1.04)

(Table 2). Continued.

Clone Name	Sequence affiliation (NCBI accession no.)[1]	Clones identified, n* (% of clones)[2]	
		Normal	Endometritis
E66	Uncultured *gamma proteobacterium* clone 16A18 (EU409846.1)	0(0)	1(1.04)
E68	Uncultured *Ruminococcaceae* bacterium clone EMP_Z35 (EU794085.1)	0(0)	1(1.04)
E69	Uncultured organism clone ELU0156-T284-S-NIPCRAMgANa_000302 (HQ805784.1)	0(0)	1(1.04)
E70	Uncultured bacterium clone calf784_10wks_grp1_A03 (GQ448705.1)	0(0)	1(1.04)
E73	Uncultured bacterium clone N27 (FJ951858.1)	0(0)	1(1.04)
E74	Uncultured bacterium clone TU1_aaa03d10 (EU470091.1)	0(0)	1(1.04)
E80	Uncultured bacterium clone calf784_6wks_grp2_E07 (GQ448612.1)	0(0)	1(1.04)
E81	Uncultured *Ruminococcaceae* bacterium clone EMP_C8 (EU794275.1)	0(0)	1(1.04)
E84	Uncultured bacterium clone DLN-152 (FJ848448.1)	0(0)	1(1.04)
E85	Uncultured bacterium clone D-1 (HQ860731.1)	0(0)	1(1.04)
E92	Uncultured bacterium clone LI142-1O6 (FJ671765.1)	0(0)	1(1.04)
E93	Uncultured bacterium clone Hmb2-28 (JX096326.1)	0(0)	1(1.04)
E94	Uncultured bacterium gene for 16S rRNA (AB506359.1)	1(1.82)	1(1.04)
N2	Uncultured bacterium clone CA_132 (JN559574.1)	3(5.45)	0(0)
N7	Uncultured *Ruminococcaceae* bacterium clone EMP_AF18 (EU794236.1)	1(1.82)	0(0)
N8	Uncultured bacterium clone gir_aah93a01 (EU775246.1)	1(1.82)	0(0)
N10	Uncultured bacterium clone SJTU_C_12_90 (EF404570.1)	1(1.82)	0(0)
N12	Uncultured bacterium clone G26 (FJ951875.1)	1(1.82)	0(0)
N14	Uncultured bacterium clone BH2_aao21d11 (EU466292.1)	1(1.82)	0(0)
N15	Uncultured *Ruminococcaceae* bacterium clone EMP_D27 (EU794190.1)	1(1.82)	0(0)
N17	Uncultured bacterium clone AS1_aao39g05.Contig1 (EU772318.1)	1(1.82)	0(0)
N18	Uncultured bacterium clone ELAND_32 (AY858498.2)	1(1.82)	0(0)
N21	Uncultured organism clone ELU0018-T230-S-NIPCRAMgANa_000531 (HQ745064.1)	1(1.82)	0(0)
N24	Uncultured bacterium clone OK3_b09_1 (EU468758.1)	1(1.82)	0(0)
N25	Uncultured bacterium clone FF_-aag84d08 (EU774958.1)	1(1.82)	0(0)
N29	Uncultured bacterium clone D-29 (AY676489.1)	1(1.82)	0(0)
N32	Uncultured rumen bacterium clone SR16 (DQ394632.1)	1(1.82)	0(0)
N33	Uncultured bacterium clone SBSD_aaa02c02 (EU475393.1)	1(1.82)	0(0)
N37	Uncultured *Ruminococcaceae* bacterium clone EMP_E02 (EU794192.1)	1(1.82)	0(0)
N38	Uncultured bacterium clone DLN-43 (FJ848395.1)	2(3.63)	0(0)
N51	Uncultured bacterium clone EMP_D46 (EU794191.1)	1(1.82)	0(0)
N52	Uncultured *Treponema* sp. clone EMP_F10 (EU794168.1)	1(1.82)	0(0)
N54	Uncultured bacterium clone TU1_aaa03f09 (EU470080.1)	1(1.82)	0(0)
N55	Uncultured bacterium clone DLN-136 (FJ848430.1)	1(1.82)	0(0)

*n is the no. of clones matched with specific bacteria or group.
[1]Most significant National Center for Biotechnology Information (NCBI) database match.
[2]Pecentage of clones in each library.

bacteria (Figure **2**). Phylogenetic analysis has shown that most of the clones were matched with *Proteobacteria* (36.42%) and is the most diversified group with 25 OTUs (Operational taxonomic units). The other clones belong to *Tenericutes* (14.56%), *Fusobacteria* (5.96%), *Firmicutes* (3.97%) and *Bacteroidetes* (1.98%). Most of the OTUs of uncultured group have identity to the groups *Firmicutes* and *Proteobacteria*. The E15 clone was more similar to *Bacteroides ureolyticus* strain R-37890 belongs to *Bacteroidetes* group according to BLAST search, but in the phylogenetic tree the OTU of clone E15 was merged in *Proteobacteria* group because of the more sequence similarity to other clones related to *Proteobacteria* (Figure **2**).

Based on BLAST search most of the 16S rRNA clone sequences from normal status library were affiliated to cultured bacteria (54.54%) and the remaining clones were affiliated to uncultured group of bacteria (45.45%). From the cultured group most of the clones were matched with the bacteria related to group *Proteobacteria* (36.36%) (Figure **2**, Table **2**) and most of the clones having similarity to the bacterium

Haemophilus felis (16.36%) (Table **2**). The remaining clones were affiliated to the groups *Fusobacteria* (9.09%), *Firmicutes* (3.63%), *Tenericutes* (3.63%) and *Bacteriodetes* (1.82%). In the *Fusobacteria* group, the *Fusobacterium* sp. CSL-7530 (5.45%) is the major bacterium to which major number of the sequences has identity (Table **2**). In the normal status clone library a large proportion of clones were matched with uncultured bacteria (45.45%), is an indication for the presence of a large number of uncultured bacteria in the postpartum normal buffaloes. The bacteria which were identified only in normal status samples were shown in the Table **3**.

From the endometritic state library, according to BLAST search, most of the 16S rRNA clone sequences were affiliated to cultured bacteria (67.71%) and rest of them were affiliated to uncultured group (32.29%). In the cultured group most of the sequences from endometritic status library were affiliated to *Proteobacteria* (36.46%) and *Tenericutes* (20.83%) (Figure **2**, Table **2**). In *Proteobacteria* group most of the sequences have identity with the bacterium *Psychrobacter pulmonis* (11.46%), *Pseudomonas psychrophila* (6.25%), *Pseudomonas* sp. P4 (2010) (6.25%) and *Psychrobacter* sp. PRwf-1 (5.21%). In the *Tenericutes* group, all the clone sequences were matched with the bacteria *Ureaplasma diversum*

Table 3: List of the Bacteria which were Identified Only in the Normal Status Uterine Samples

Clone name	Sequence affiliation (NCBI accession no.)
N40	*Lachnospiraceae bacterium canine* oral taxon 037 clone OD066 **(JN713203.1)**
N30	*Clostridium nexile* DSM 1787 **(NR_029248.1)**
N3	*Pasteurellaceae bacterium Baika3* **(HM626621.1)**
N4	*Pasteurella mairii* strain 9801/75 **(AY431032.1)**
N9	*Haemophilus felis* strain ATCC49733 **(NR _025073.1)**
N6	*Aggregatibacter segnis canine* oral taxon 093 clone OE003 **(JN713257.1)**
N44	*Pasteurella canis canine* oral taxon 273 clone ZJ072 **(JN713438.1)**
N45	*Haemophilus parasuis* strain HS1079 **(FJ667960.1)**
N47	*Actinobacillus seminis* strain CCUG 27187 **(NR_042872.1)**
N48	*Bisgaard* Taxon 17 **(AF024529.1)**
N26	*Campylobacter* sp. canine oral taxon 011 clone ZJ010 **(JN713171.1)**
N34	*Campylobacter concisus* strain UNSWCD **(GQ167662.1)**
N27	*Neisseria canis canine* oral taxon 137 clone OK030 **(JN713302.1)**
N1	*Fusobacterium* sp. CSL-7530 **(EU597748.1)**
N19	*Streptobacillus* sp. canine oral taxon 370 clone 2B078 **(JN713542.1)**
N2	Uncultured bacterium clone CA_132 **(JN559574.1)**
N7	Uncultured *Ruminococcaceae* bacterium clone EMP_AF18 **(EU794236.1)**
N8	Uncultured bacterium clone gir_aah93a01 **(EU775246.1)**
N10	Uncultured bacterium clone SJTU_C_12_90 **(EF404570.1)**
N12	Uncultured bacterium clone G26 **(FJ951875.1)**
N14	Uncultured bacterium clone BH2_aao21d11 **(EU466292.1)**
N15	Uncultured *Ruminococcaceae* bacterium clone EMP_D27 **(EU794190.1)**
N17	Uncultured bacterium clone AS1_aao39g05.Contig1 **(EU772318.1)**
N18	Uncultured bacterium clone ELAND_32 **(AY858498.2)**
N21	Uncultured organism clone ELU0018-T230-S-NIPCRAMgANa_000531 **(HQ745064.1)**
N24	Uncultured bacterium clone OK3_b09_1 **(EU468758.1)**
N25	Uncultured bacterium clone FF_-aag84d08 **(EU774958.1)**
N29	Uncultured bacterium clone D-29 **(AY676489.1)**
N32	Uncultured rumen bacterium clone SR16 **(DQ394632.1)**
N33	Uncultured bacterium clone SBSD_aaa02c02 **(EU475393.1)**
N37	Uncultured *Ruminococcaceae* bacterium clone EMP_E02 **(EU794192.1)**
N38	Uncultured bacterium clone DLN-43 **(FJ848395.1)**
N51	Uncultured bacterium clone EMP_D46 **(EU794191.1)**
N52	Uncultured *Treponema* sp. clone EMP_F10 **(EU794168.1)**
N54	Uncultured bacterium clone TU1_aaa03f09 **(EU470080.1)**
N55	Uncultured bacterium clone DLN-136 **(FJ848430.1)**

(20.83%) (Table **2**). Some of the clone sequences were affiliated with the groups of bacteria *Firmicutes* (4.16%), *Fusobacteria* (4.16%) and *Bacteroidetes* (2.08%). Even though a large number of clones were affiliated to culture bacteria, some of the clone sequences from endometritic library were affiliated to uncultured group of bacteria (32.29%) which is revealing the presence of a moderate portion of uncultured bacteria in the uteri of the postpartum endometritic buffaloes (Table **2**). The bacteria identified only in the endometritic status samples were shown in Table **4** and the bacteria identified both in normal and endometrtic samples were shown in Table **5**.

The Z-test values and p-values of the same groups of postpartum normal and endometritic clone library were shown in Table **6**. According to the p-values obtained from the Z-test, *Tenericutes* is group of bacteria which was significantly (P<0.0001) present in the endometritic status library when compared to normal status library (Table **6**) but the uncultured group of bacteria was significantly (P=0.0096) present in the normal status library compared with endometritic status library (Table **6**). The p-values for the bacteria identified in both normal and endometritic clone library were also shown in the Table **5**. The bacteria *Ureaplasma diversum* and *Psychrobacter pulmonis* were significantly (P<0.0001) present in the postpartum endometritic uterine samples (Table **5**).

DISCUSSION

In India, water buffalo (*Bubalus bubalis*) is the major animal in the production of milk. The postpartum infection incidents were high in buffaloes than cows [3, 13]. The metagenomic analysis by culture independent methods like 16S rRNA gene cloning and pyrosequencing of the 16S rRNA gene shown the

Table 4: List of the Bacteria which were Identified only in the Endometritic Status Uterine Samples

Clone name	Sequence affiliation (NCBI accession no.)
E75	*Sphingobacterium* sp. HaLB8 **(HM352374.1)**
E17	*Streptococcus uberis* 0140J **(AM946015.1)**
E32	*Filifactor alocis canine* oral taxon 001 clone OB055 **(JN713152.1)**
E10	*Psychrobacter* sp. PRwf-1 **(CP000713.1)**
E59	*Psychrobacter* sp. 22 **(FJ613604.1)**
E61	*Psychrobacter* sp.BSw21684 **(JQ069959.1)**
E72	*Pseudomonas psychrophila* strain HA-4 **(JQ968688.1)**
E77	*Psychrobacter faecalis* strain SCSGAB0010 **(JX315290.1)**
E78	*Pseudomonas* sp. P4 (2010) **(HM196356.1)**
E82	*Yersinia similis* partial 16S rRNA gene, strain Y239 **(AM182407.1)**
E88	*Yersinia pestis* A1122 **(CP002956.1)**
E18	*Campylobacter hominis* ATCC BAA-381 **(CP000776.1)**
E2	*Fusobacterium varium* **(AB640694.1)**
E1	Uncultured bacterium clone EAC_1aaa02e08 **(EU774679.1)**
E24	Uncultured bacterium clone IR aaa02h01 **(EU474649.1)**
E35	Uncultured bacterium clone A-18 **(HQ860486.1)**
E40	Uncultured bacterium clone 1103200832522 **(EU845713.1)**
E60	Uncultured bacterium clone 1103200828900 **(EU845467.1)**
E63	Uncultured bacterium clone HWGB-66 **(JQ684324.1)**
E64	Uncultured bacterium clone KO1_aai44c07 **(EU461105.1)**
E65	Uncultured bacterium clone BH2_aao23c02 **(EU466407.1)**
E67	Uncultured bacterium clone calf32_10wks_grp1_F02 **(GQ448226.1)**
E66	Uncultured *gamma proteobacterium* clone 16A18 **(EU409846.1)**
E68	Uncultured *Ruminococcaceae* bacterium clone EMP_Z35 **(EU794085.1)**
E69	Uncultured organism clone ELU0156-T284-S-NIPCRAMgANa_000302 **(HQ805784.1)**
E70	Uncultured bacterium clone calf784_10wks_grp1_A03 **(GQ448705.1)**
E73	Uncultured bacterium clone N27 **(FJ951858.1)**
E74	Uncultured bacterium clone TU1_aaa03d10 **(EU470091.1)**
E80	Uncultured bacterium clone calf784_6wks_grp2_E07 **(GQ448612.1)**
E81	Uncultured *Ruminococcaceae* bacterium clone EMP_C8 **(EU794275.1)**
E84	Uncultured bacterium clone DLN-152 **(FJ848448.1)**
E85	Uncultured bacterium clone D-1 **(HQ860731.1)**
E92	Uncultured bacterium clone Ll142-1O6 **(FJ671765.1)**
E93	Uncultured bacterium clone Hmb2-28 **(JX096326.1)**

Table 5: The p-Values of the Bacterial Clones that were Commonly Found in Both Normal and Endometritic Status Clone Libraries

Clone name	Sequence affiliation (NCBI accession no.)	two-tailed p-value
E3	*Ureaplasma diversum* strain T95 **(JN935894.1)**	< 0.0001
E4	*Ureaplasma diversum* strain A417 **(NR_025878.1)**	< 0.0001
E15	*Bacteroides ureolyticus* strain R-37890 **(FN401327.1)**	0.5675
E42	*Psychrobacter pulmonis* strain KOPRI24933 **(EF101551.1)**	< 0.0001
E94	Uncultured bacterium gene for 16S rRNA **(AB506359.1)**	0.5675

Table 6: The Z-Test Values and the p-Values of Different Bacterial Groups Obtained from Normal and Endometritic Clone Libraries

S. No.	Bacterial group	One proportion Z value	two-tailed p-value
1.	*Bacteroidetes*	0.1906	0.8489
2.	*Firmicutes*	0.2776	0.7813
3.	*Proteobacteria*	0.0204	0.9837
4.	*Tenericutes*	9.0103	< 0.0001
5.	*Fusobacteria*	-1.6803	0.0929
6.	Uncultured	-2.5896	0.0096

presence of a large number bacterial population in the postpartum uteri of the cows [16, 22, 23]. Some of the bacteria present in the uteri of the buffaloes after two to four weeks of parturition were identified by culture dependent methods. *E. coli*, *S. aureus* and *S. pyogenes* are most predominant isolates from the uteri of postpartum buffaloes [5]. But there are no culture independent studies for the identification of large number of bacterial population in the uteri of the postpartum infected buffaloes. In the present study a culture dependent method (16S rRNA gene cloning) was used to identify and compare the phylogenetic profile of the intrauterine microbita of postpartum normal and endometritic buffaloes. The sequence of 16S rRNA gene fragments were obtained by cloning and sequencing of 16S rRNA gene from the bacterial DNA isolated from the uterine fluid of postpartum buffaloes. BLAST search of these 16S rRNA gene sequences obtained from clone libraries of normal and endometritic postpartum buffaloes were revealed that the clone libraries were belongs to five known culture groups and an uncultured group of bacteria.

From the normal status library most of the clones related to the cultured groups like *Proteobacteria* and *Fusobacteria*, but many clones were having less identity with the cultured bacteria, because may be these sequences were belongs to groups of bacteria which were uncultured so far or might represent new bacterial branches not related, or only distantly related, to known cultured microorganisms. From the cultured group of clone sequences, most of them were affiliated to the bacteria *Haemophilus felis* and *Fusobacterium* sp. CSL-7530. *Haemophilus felis* is a potent pathogen for cats causing upper respiratory tract infections. Even though the bacterial strains related to *Haemophilus* genus are causing the reproductive diseases in cow [25], there are no reports regarding to role of *Haemophilus felis* strain in reproduction. The role of *Fusobacterium* genus in various human and cattle diseases was reported [26], but the involvement of *Fusobacterium* sp. CSL-7530 strain in diseases were not reported.

Most of the clone sequences from endometrtic status library were affiliated to known cultured bacteria belongs to *Proteobacteria* and *Tenericutes* groups. But a major number of the clones from endometrtic status library also have identity with the uncultured group of bacteria. This was telling that may be a large portion of uncultured bacteria is present in the uteri of postpartum endometritic buffaloes. From the known cultured group

of sequences, most of the clones have identity with the bacteria *Psychrobacter pulmonis*, *Pseudomonas psychrophila* and *Pseudomonas* sp. P4 (2010) belongs to the group *Proteobacteria*. *Psychrobacter pulmonis* was first isolated from the lungs of lambs [27]. Even though the effect of *Psychrobacter pulmonis* on reproduction was not reported, we have been observed that this is one of the bacteria present highly in the intrauterine of the endometritic buffaloes. The bacterium belongs to the genus *Pseudomonas* were commonly nosocomial infections. The role of *Pseudomonas psychrophila* and *Pseudomonas* sp. P4 (2010) in the reproduction related diseases was not known. Additionally, sequences from endometrtic status library have identity with *Ureaplasma diversum* belongs to the group *Tenericutes*. We have identified the presence of two *Ureaplasma diversum* strains (*Ureaplasma diversum* strain A417 and *Ureaplasma diversum* T95) in the uterine samples of postpartum endometritic buffaloes. The reports have shown that the *Ureaplasma diversum* is one of the important members of the *Mycoplasmataceae* family which is a potent cause for postpartum infertility in cattle [28]. *U. diversum* species have both pathogenic and non-pathogenic strains. This was considered as a normal microfloral inhabitant of the lower reproductive tract of females [29], but it has also been associated with various forms of reproductive failure in cattle [28], including granular vulvovaginitis, endometritis, salpingitis, early embryonic death, weak calves, decreased conception rates, balanoposthitis, impaired spermatozoids [29-31] and seminal vesiculitis in bulls [32].

Metagenomics brought new perception about the structure, metabolism, and evolution of uncultured organisms occupying diverse niches [13, 14]. This was given the impotence to investigate uterine microbiota with culture independent methods. The precious metagenomic studies have been done by using various culture independent methods to identify the intrauterine bacteria of postpartum cows and were shown the presence of different types of bacterial population in the uteri of the cows [15, 22, 23]. According to our knowledge there were no studies to identify the intrauterine bacterial population of postpartum buffaloes by using culture independent methods. Here we are submitting the first report regarding to metagenomic analysis (cloning and sequencing of 16S rRNA gene fragments) of uterine microbiota of postpartum normal and endometritic buffaloes. The major aim of our study was to analyze the variation between the composition and community of bacteria in the uterus of the postpartum buffaloes and their role on health of animal by using culture-independent methods.

Based on our results we observed that the bacterial community of normal and endometritic status buffaloes was varying largely. Only few bacteria were present commonly in both types of animals. Even though our data is not sufficient to decide the full status of the animal, may be this is one of the platform to investigate the full profile of microbiota in the postpartum buffaloes uterus. Use of the high-throughput methods may also help to reach a consensus and define what constitutes or determine a pathogenic bacteria community in this syndrome.

CONCLUSION

The present study has shown the presence of various types of bacteria in the postpartum normal and endometritic buffaloes and also shown the complexity of bacterial community of the normal status buffaloes and the buffaloes suffering with endometritis. The 16S rRNA clone libraries were affiliated with five known cultured groups and an uncultured bacterial group. *Proteobacteria* was the predominant group in both normal and endometritic clone libraries. The group *Tenericutes* was also one of the dominant groups in endometritic clone library. *Ureaplasma diversum* which belongs to *Tenericutes* group was significantly present in endometritic clone library which was causing the reproductive problems in cattle.

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